Intracellular Features of Type II Procollagen and Chondroitin Sulfate Proteoglycan Synthesis in Chondrocytes

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The intracellular compartments of chondrocytes involved in the synthesis and processing of type II procollagen and chondroitin sulfate proteoglycan (CSPG) monomer were investigated using simultaneous double immunofluorescence and lectin localization reactions. Type II procollagen was distributed in vesicles throughout the cytoplasm, whereas intracellular precursors of CSPG monomer were accumulated in the perinuclear cytoplasm. In this study, cytoplasmic vesicles that stained intensely with antibodies directed against CSPG monomer but did not react with type II collagen antibodies, also were observed. A monoclonal antibody, 5-D-4, that recognizes keratan sulfate determinants was used to identify the Golgi complex (the site of keratan sulfate chain elongation). Staining with 5-D-4 was restricted to the perinuclear cytoplasm. The vesicles outside the perinuclear cytoplasm that stained intensely with antibodies to CSPG monomer did not react with 5-D-4. Fluorescent lectins were used to characterize further subcellular compartments. Concanavalin A, which reacts with mannose-rich oligosaccharides, did not stain the perinuclear region, but it did stain vesicles throughout the rest of the cytoplasm. Because mannose oligosaccharides are added cotranslationally, the stained vesicles throughout the cytoplasm presumably correspond to the rough endoplasmic reticulum. Wheat germ agglutinin, which recognizes N-acetyl-D-glucosamine and sialic acid (carbohydrates added in the Golgi), stained exclusively the perinuclear cytoplasm. By several criteria (staining with the monoclonal antibody 5-D-4 and with wheat germ agglutinin), the perinuclear cytoplasm seems to correspond to the Golgi complex. The cytoplasmic vesicles that react with anti-CSPG monomer and not with anti-type II collagen contain precursors of CSPG monomer not yet modified by Golgi-mediated oligosaccharide additions (because they are not stained with wheat germ agglutinin or with the anti-keratan sulfate antibody); these vesicles may have a unique function in the processing of CSPG.

The extensive extracellular matrix of hyaline cartilage tissue is composed mainly of fibrous type II collagen [1] and large, link-stabilized aggregates of chondroitin sulfate proteoglycan (CSPG) monomer, and hyaluronic acid [reviewed in 2–6]. The elaboration of this characteristic extracellular matrix is the result of many synthetic, processing, and assembly events. Intracellular and extracellular steps are involved. In the case of the 1–5×10^6 Da CSPG monomer of cartilage, for example, the core protein represents only 8–10% of the macromolecule; O-linked carbohydrate chains of chondroitin sulfate and keratan sulfate, asparagine-linked oligosaccharides, and other complex carbohydrates make up the remainder [2–7]. The biosynthesis of type II procollagen is characterized also by numerous co- and posttranslational processing steps, such as the hydroxylation of proline and lysine, glycosylation of asparagine and hydroxylysine, formation of interchain disulfide bonds, and assembly of triple helical molecules [8–10]. The assembly of proteoglycan aggregates, the enzymatic conversion of procollagen to collagen, and the formation of collagen fibers are extracellular events.

Clearly, the rough endoplasmic reticulum and Golgi complex, and perhaps other cytoplasmic membrane-bound compartments, are involved in the intracellular modifications of these complex matrix molecules. The presence of a highly developed, rough endoplasmic reticulum and Golgi complex is a major ultrastructural feature of the cartilage cell [11]. To some degree, the role of membrane-bound compartments in the biosynthesis of type II procollagen and CSPG has been documented by autoradiographic studies [12,13] and by the characterization of subcellular fractions [14–19]. For example, sulfation has been shown to be a Golgi-mediated modification. However, other details of the synthesis, processing, and assembly of both type II collagen and CSPG in chondrocytes and the characterization of subcellular organelles involved in these events remain to be clarified.

Both type II collagen and CSPG monomer are characterized by covalently attached oligosaccharides, as discussed above. Presumably, then, precursor processing could be traced through the progressive modifications of these oligosaccharides. It has been shown that mannose-rich sugars are added cotranslationally to newly synthesized proteins [20]. Subsequently, mannose-rich sugars are trimmed and may be converted to more complex oligosaccharides. The synthesis and processing of these oligosaccharides are accomplished in distinct intracellular compartments; cotranslational addition of mannose-rich sugars to asparagine residues occurs in the rough endoplasmic reticulum whereas later modifications occur in several steps in the Golgi [20–22]. Lectins have proved to be very useful in the characterization of oligosaccharides because they bind to specific sugars [23]. Reactivity with labeled lectins has been used in many studies for the identification of subcellular compartments containing oligosaccharide moieties of interest [24]. For example, concanavalin A will react with the rough endoplasmic reticulum because this lectin binds to the mannose-rich oligosaccharides added cotranslationally. Fluorescent-labeled lectins have been used to identify cytoplasmic compartments of cultured cells in the light microscope [25].

To learn more about the intracellular compartments involved in the synthesis of type II procollagen and CSPG, simultaneous double immunofluorescence and lectin localizations were used to characterize cytoplasmic structures in the same individual chondrocytes. Chondrocytes in monolayer culture are well suited to this study because they contain intracellular compartments that are observed easily in the light micro-

scope. Previous work documented the synthesis and deposition of extracellular matrix by chondrocytes as a function of time and condition of culture [26–31]. Some features of intracellular processing are reported in recent studies [29–32]. Results of the present study indicated that the processing, modification, or assembly of different classes of matrix constituents occur at the same time in separate cytoplasmic compartments of the same cartilage cells. Interestingly, unusual cytoplasmic vesicles were detected that apparently contain precursors of CSPG monomer not yet modified by Golgi-mediated oligosaccharide additions, but which do not contain detectable type II procollagen.

MATERIALS AND METHODS

Materials

Fertile Rhode Island Red chicken eggs were obtained from Webster Poultry Farms (Auburn, NY). Tissue culture plates were products of Corning (Corning, NY). Trypsin, Ham's F-12 medium, fetal calf serum, antibiotic-antimycotic mixture, and Hank's balanced salt solution (HBSS) were obtained from Grand Island Biological Supply Co. (Grand Island, NY). Nonidet P-40 was obtained from Bethesda Research Labs (Rockville, MD). Testicular hyaluronidase was purchased from Leo (Helsingborg, Sweden). Wheat germ agglutinin and concanavalin A coupled to fluorescein isothiocyanate (FITC) or to tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Vector Laboratories (Burlingame, CA). Goat anti-guinea pig IgG, goat anti-rabbit IgG, and goat anti-mouse IgG conjugated to FITC or TRITC were obtained from Cappel Laboratories (Cochranville, PA). The mouse monoclonal antibody 5-D-4, which recognizes keratan sulfate, was generously given to us by Drs. Bruce Caterson, James Christner, and John Baker.

Cell Culture

Chondrocytes were obtained from the sterna of 14–16-day-old chicken embryos using a modification of the procedure of Cahn et al [33]. Cells were plated on gelatinized, carbon-coated coverslips at a concentration of 2×10^5 cells per 60 mm tissue culture dish in 3 ml of Ham's F-12 medium containing 9% fetal calf serum and 1% antibiotic-antimycotic mixture. Cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2. Culture medium was changed every other day.

In previous studies, the authors found that the deposition of extracellular type II collagen interfered with the analysis of intracellular compartments containing collagen and CSPG precursors [26–31]. Therefore, in order to prevent the deposition of extracellular type II collagen, the culture medium was not supplemented with ascorbate. Ascorbate is a co-factor necessary for the hydroxylation of proline, a posttranslational modification that is important for the stability of triple helical collagen molecules and subsequent extracellular collagen fiber formation [8,9,34]. In the absence of ascorbate supplements, type II procollagen is synthesized and secreted by chondrocytes but is not deposited extracellularly.

Coverslips were removed at 5 days of culture, rinsed several times with HBSS, and fixed in 75% ethanol. In some experiments, chondrocytes were incubated with testicular hyaluronidase for 10 min at 37°C, and washed several times prior to fixation, as described previously [26].

Immunofluorescent Staining and Lectin Localization

Fixed cells on coverslips were rinsed with 75% ethanol, treated for 2 min with 98% ethanol/ether (1:1 vol/vol), and air dried [35]. Alternatively, cells fixed in 75% ethanol were re-equilibrated in HBSS and treated for 15 to 20 min with 1% Nonidet P-40 in HBSS to permeabilize the cells.

Subsequently, permeabilized cells were reacted for 20 min at room temperature with the polyclonal rabbit and guinea pig antibodies described below. Following washes in HBSS, the cells were reacted for 20 min with goat anti-rabbit IgG and goat anti-guinea pig IgG coupled to either FITC or TRITC. Double immunofluorescence reactions using monoclonal antibodies in combination with polyclonal antibodies were performed as described by Vertel and Barkman [29].

Alternatively, permeabilized cells were incubated for 20 min with concanavalin A and/or wheat germ agglutinin coupled to either FITC or TRITC. In some cases, cells were reacted with lectins and antibodies.

After further washes, the coverslips were mounted in phosphate buffer/glycerol (1:9, vol/vol) and viewed and photographed using a Leitz Ortholux microscope equipped with phase and epifluorescence optics and with specific filters for the visualization of fluorescein and rhodamine fluorescence. The same fields of cells were selected from double-stained specimens and photographed sequentially for FITC and TRITC staining and, in some cases, under phase. Fluorescent localization patterns described in Results are based on the analysis of a minimum of 50 independently stained specimens.

Antibodies

Polyclonal rabbit and guinea pig antibodies directed against the following antigens were prepared and characterized previously: 1) hyaluronidase-digested CSPG monomer isolated from the epiphyses of 14-day-old chicken embryos [26,36] and 2) pepsin-extracted type II collagen isolated from adult chicken sterna [26,36]. The mouse monoclonal antibody, 5-D-4, which recognizes keratan sulfate determinants of CSPG, was characterized by Caterson et al [37] and used previously for immuno-fluorescent localization [29].

RESULTS

The localization of type II collagen and CSPG was examined in sternal chondro-cytes using double immunofluorescent staining reactions. In previous studies, it was established that the antibodies directed against type II collagen and CSPG monomer react with these matrix molecules at all stages of their biosynthesis: 1) by immuno-fluorescent reaction with intracellular biosynthetic intermediates and with the highly modified, completed products in extracellular cartilage matrix [26–28,30,31]; and 2) by immunoprecipitation of the corresponding unmodified polypeptide precursors synthesized in cartilage RNA-directed cell-free translation reactions [36,38]. Thus, these antibodies can be used to investigate all stages in the synthesis and processing of type II collagen and CSPG monomer. Because it has been established that the conversion of procollagen to collagen is an extracellular event [8–10], all intracellular forms of collagen are referred to as procollagen (even though the antibodies originally were prepared against type II collagen and cannot be used to distinguish procollagen from collagen).

In the first section of Results, the intracellular distribution of type II procollagen is compared with that of CSPG precursors, utilizing the antibodies just described. In the second section of Results, the intracellular distribution of a monoclonal antibody that recognizes keratan sulfate glycosaminoglycans is compared with that of the polyclonal antibodies described above. Because it has been established that the synthesis of sulfated glycosaminoglycan chains occurs in the Golgi complex, the keratan sulfate monoclonal antibody was used to distinguish the Golgi complex from other cytoplasmic compartments. Finally, the use of fluorescent-labeled lectins in the further characterization of intracellular compartments is described in the third section of Results. Concanavalin A was used to identify the rough endoplasmic reticulum because it binds to mannose-rich oligosaccharides that are added cotranslationally. Wheat germ agglutinin was used to identify the Golgi complex because it recognizes N-acetyl-D-glucosamine and sialic acid, carbohydrates that are added in the Golgi.

The staining patterns of unfixed cells and fixed, nonpermeabilized cells (which are stained only extracellularly) were compared with those of fixed, permeabilized cells (which are stained both intracellularly and extracellularly) in order to distinguish intracellular and extracellular localization (not shown). The patterns of intracellular staining observed were similar, whether cells were fixed with 75% ethanol and permeabilized by ethanol-ether treatment or detergent as described in Materials and Methods, or whether cells were fixed with paraformaldehyde or glutaraldehyde and detergent permeabilized. Therefore, it was assumed that the intracellular localizations reported reflect the biosynthetic properties of individual cells and do not result from fixation artifacts.

Simultaneous Immunolocalization of Type II Collagen and CSPG

The simultaneous localization in chondrocytes of antibodies against type II collagen and CSPG is shown in Figure 1. The chondrocytes in the colony shown in the phase micrograph (Fig. 1a) are surrounded by an abundant extracellular matrix of CSPG (Fig. 1b). Each chondrocyte contains intracellular type II procollagen in discrete cytoplasmic compartments (Fig. 1c). Extracellular type II collagen was not deposited because the culture medium was not supplemented with ascorbate (see Materials and Methods). For many chondrocytes, staining with type II collagen antibodies was observed both in the perinuclear region and throughout the rest of the cytoplasm. A few chondrocytes exhibit type II collagen immunoreactivity restricted, or virtually restricted, to the perinuclear cytoplasm (arrows, Fig. 1c). The particular distribution of intracellular type II procollagen may vary within one culture, or, as shown in Figure 1, within one group of chondrocytes. Most commonly, type II procollagen-containing vesicles were observed to be distributed throughout the cytoplasm. However, in some samples, the majority of chondrocytes contained type II procollagen concentrated in the perinuclear cytoplasm. Flattened fibroblasts in the same culture did not react with either of these antibodies (asterisks, Fig. 1).

The presence of considerable extracellular CSPG made it difficult to observe intracellular CSPG monomer. However, in the absence of deposited, fibrillar, extracellular type II collagen (as in these cultures grown without ascorbate supplements), it is possible to remove extracellular CSPG by enzymatic digestion prior to fixation and subsequently to observe intracellular reaction product. The enzymatic digestion
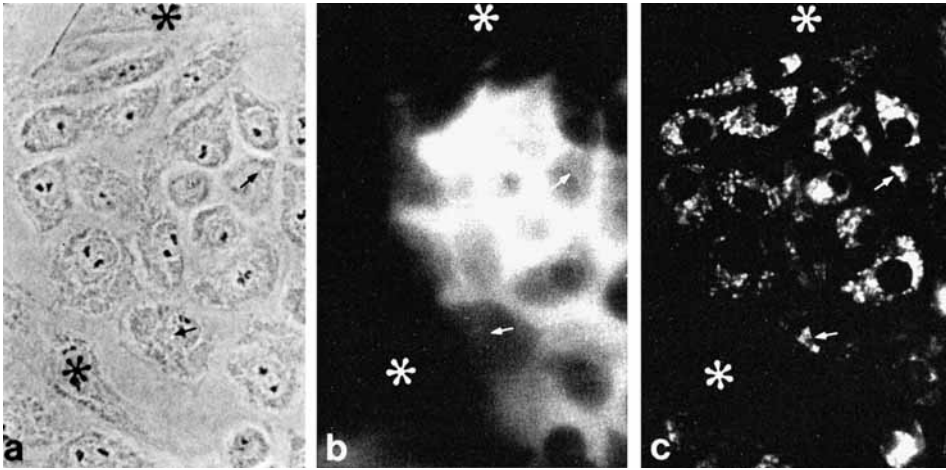
Fig. 1.   Simultaneous immunofluorescent localization of CSPG and type II collagen in chondrocytes. Sternal chondrocytes in culture 5 days were fixed directly in 75% ethanol and processed for double immunofluorescent staining as described in Materials and Methods. Cells on coverslips were incubated simultaneously with rabbit anti-CSPG monomer and guinea pig anti-type II collagen, followed by incubation with FITC-coupled goat IgG anti-rabbit IgG and TRITC-coupled goat IgG anti-guinea pig IgG. The same double-stained field is shown in phase (a) and for the simultaneous immunolocalization of CSPG (b) and type II collagen (c). Extracellular CSPG (b) surrounds the chondrocytes which contain intracellular type II procollagen (c). Many chondrocytes contain type II procollagen distributed throughout the cytoplasm (c). Two cells containing predominant type II collagen staining of the perinuclear cytoplasm (c) are indicated by arrows. Asterisks denote flattened fibroblasts which do not stain with either of the antibodies. Magnification ×500.

of hyaluronic acid and chondroitin sulfate glycosaminoglycans with testicular hyaluronidase resulted in the release of CSPG monomers so that they could be washed away with buffer. Subsequently, intracellular CSPG was observed in permeabilized chondrocytes and compared directly with intracellular type II procollagen in the same cells (Fig. 2). The phase micrograph (Fig. 2a) of the double-stained field shown in Figure 2 reveals the presence of nonstaining, flattened cells in addition to the chondrocytes. Although both type II procollagen and CSPG monomer precursors were detected in the same chondrocytes, the pattern of intracellular localization of CSPG monomer (Fig. 2b) differed from that of type II procollagen (Fig. 2c). The intracellular distribution of type II procollagen remained unchanged after hyaluronidase digestion; most chondrocytes contained type II procollagen in separate vesicles throughout the cytoplasm and in the perinuclear cytoplasm. In contrast, CSPG monomer was relatively concentrated in the perinuclear cytoplasm and in discrete vesicles that stained intensely with antibodies directed against CSPG monomer. Although it was difficult to detect intracellular CSPG monomer in non-hyaluronidase-digested samples, it was determined that the distribution of intracellular CSPG precursors was the same for digested and nondigested samples (not shown).

The intracellular compartments containing type II procollagen and CSPG monomer are compared at higher magnification in Figure 2d and e for three cells of the field shown in Figure 2a-c. Two cells contain vesicles throughout the cytoplasm that reacted with type II collagen antibodies. Immunoreactivity was not detected in the
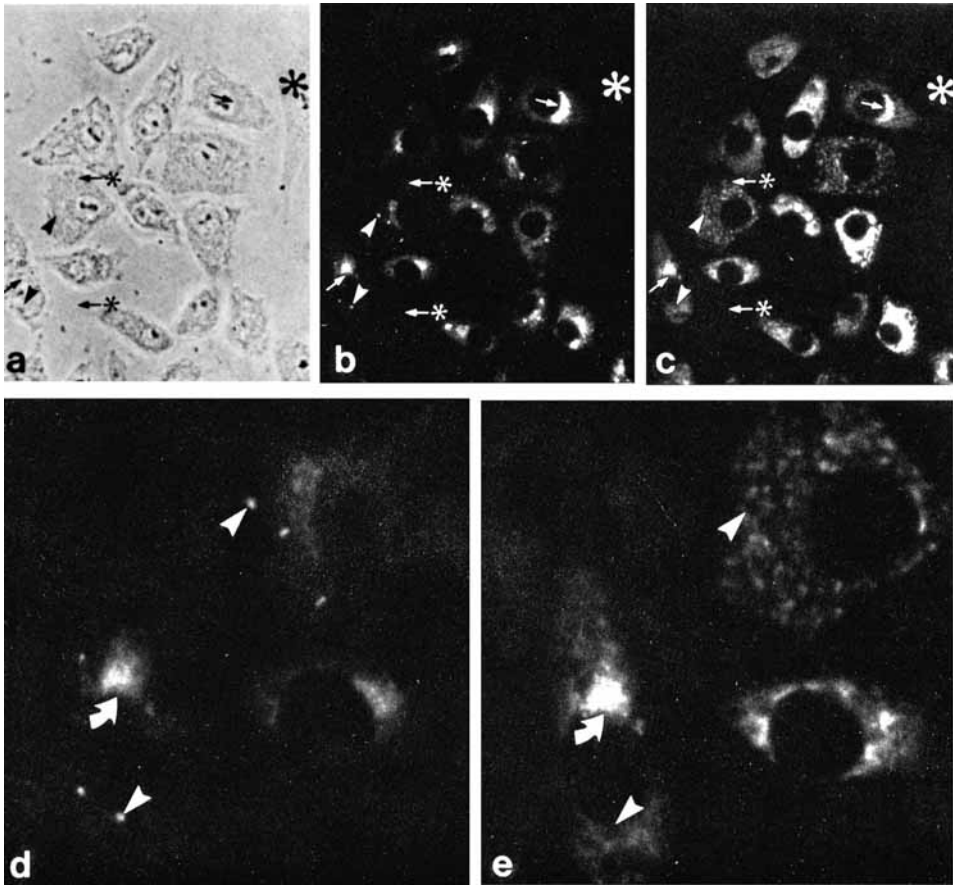
Fig. 2. Simultaneous intracellular immunolocalization of CSPG and type II collagen precursors in hyaluronidase-digested chondrocytes. Chondrocytes were grown in culture for 5 days, digested with testicular hyaluronidase, fixed, and treated for immunofluorescent staining as described in Materials and Methods. Immunofluorescent staining was performed using antibodies against CSPG monomer and type II collagen as described for Figure 1. A corresponding phase micrograph of the double-stained field is shown in (a). The simultaneous immunolocalization of CSPG monomer (b,d) and type II collagen (c,e) is compared in the same cells. A selected area which is indicated by small asterisks and arrows in a–c, and which contains three chondrocytes, is shown at higher magnification in d and e. Both type II procollagen and CSPG monomer precursors are localized in the same cells, but each has a characteristic intracellular distribution. CSPG is relatively concentrated in the perinuclear cytoplasm (b), while type II procollagen is localized in vesicles throughout the cytoplasm (c). Arrows indicate the perinuclear regions of two chondrocytes which stain intensely in that region for both CSPG (b,d) and type II collagen (c,e). Arrowheads point to discrete vesicles which stain intensely with anti-CSPG monomer (b,d), but which do not stain with anti-type II collagen (c,e). Large asterisks (a,b,c) denote flattened fibroblasts which do not stain with either of the antibodies. Magnification (a,b,c), ×500; magnification (d,e), ×1480.

perinuclear cytoplasm of these two cells. In contrast, the third cell of the field reacted with type II collagen antibodies primarily in the perinuclear cytoplasm (arrow, Fig. 2e). As shown, a different pattern of immunolocalization was observed with antibodies directed against CSPG monomer (Fig. 2d). For CSPG, immunoreactivity was noted in the perinuclear cytoplasm of all three cells. In addition, several vesicles that
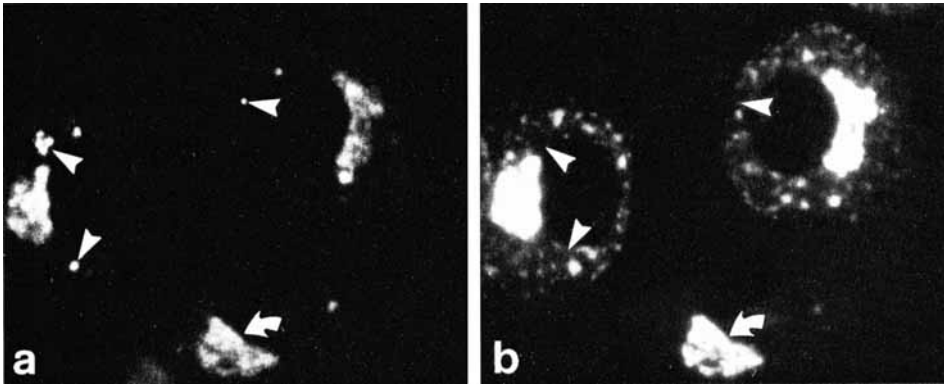
Fig. 3. Double immunofluorescent staining of hyaluronidase-digested chondrocytes with CSPG monomer and type II collagen antibodies. Chondrocytes were treated and stained with CSPG monomer and type II collagen antibodies as described for Figure 2. The same double-stained field is shown in (a) and (b). For the three chondrocytes in this field, both CSPG monomer (a) and type II procollagen (b) are concentrated in the juxtanuclear cytoplasm (arrows). Arrowheads indicate vesicles which stain intensely with anti-CSPG monomer (a), but which do not stain with type II collagen antibodies (b). Other cytoplasmic vesicles contain type II procollagen and not CSPG precursors. Magnification, ×1500.

stained intensely with antibodies to CSPG monomer, but did not stain with antibodies to type II collagen, were observed in two of the three cells (arrowheads, Fig. 2d).

Another comparison of the immunolocalization of type II collagen and CSPG monomer is made in Figure 3. Type II collagen staining was concentrated in the perinuclear region of most chondrocytes in this particular culture. Thus, both type II procollagen and CSPG are relatively concentrated in the juxtanuclear cytoplasm. Some other vesicles throughout the cytoplasm contained type II procollagen but did not react with antibodies to CSPG monomer. Cytoplasmic vesicles of approximately 500–700 nm, containing concentrated precursors to CSPG monomer but no detectable type II procollagen, were observed (arrowheads, Fig. 3). Interestingly, such vesicles exhibited intense reactivity with link protein antibodies [not shown, but see reference 30].

Vesicles that contained precursors to CSPG monomer and link, but no detectable type II procollagen, were observed in all of over 50 separate chondrocyte preparations. However, the extent to which they occurred was variable. Within any one culture, some chondrocytes contained no such vesicles, others contained one or two, and yet others contained as many as 8 to 10. Furthermore, it was noted that in some cultures vesicles were observed in many chondrocytes, whereas for others, vesicle occurrence was a rarer phenomenon.

Immunolocalization of Keratan Sulfate Determinants

The mouse monoclonal antibody 5-D-4 was used to characterize further the cytoplasmic compartments of chondrocytes. This antibody was expected to react with constituents of the Golgi complex, as keratan sulfate glycosaminoglycans are added to the CSPG core protein in the Golgi, and the antigenic determinants recognized by
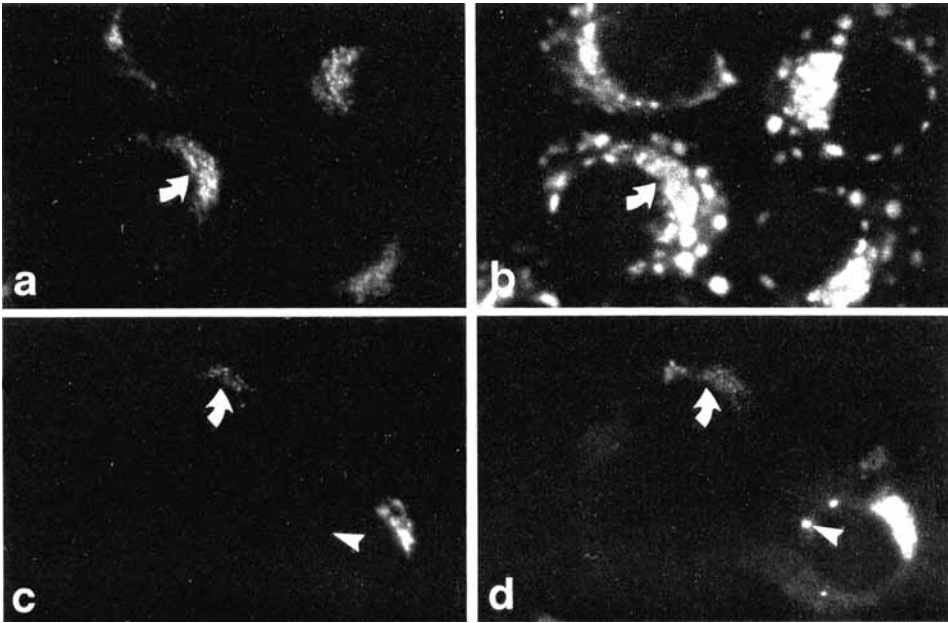
Fig. 4. Simultaneous immunofluorescent localization of a keratan sulfate monoclonal antibody and polyclonal type II collagen or CSPG monomer antibodies. Chondrocytes were cultured for 5 days and digested with testicular hyaluronidase prior to fixation and treatment for immunofluorescent localization reactions, as described in Materials and Methods. After permeabilization, cells on coverslips were incubated with antibodies in the following order: 1) Mouse monoclonal 5-D-4; 2) TRITC-coupled goat IgG anti-mouse IgG; 3) guinea pig polyclonal anti-type II collagen (a,b) or anti-CSPG monomer (c,d); 4) FITC-coupled goat IgG anti-guinea pig IgG. One set of cells was double-stained for keratan sulfate (a) and type II collagen (b). A second preparation of cells was double-stained for keratan sulfate (c) and CSPG monomer (d). In each case, the field on the left (a,c) corresponds identically to the field on the right (b,d). The immunolocalization of keratan sulfate was strictly perinuclear, as indicated by arrows (a,c). Type II collagen antibodies stained the same perinuclear cytoplasm (arrow, b) but in addition stained numerous vesicles throughout the cytoplasm of the same cells (b). CSPG monomer antibodies reacted with the perinuclear cytoplasm (arrow, d) and, in some chondrocytes, with a few discrete cytoplasmic vesicles which did not react with keratan sulfate antibodies (arrowheads, c,d). Magnifications (a,b), ×1920; magnifications (c,d), ×1600.

this monoclonal antibody are keratan sulfate glycosaminoglycans. Only the perinuclear cytoplasm was stained specifically with this monoclonal antibody (arrows, Fig. 4a, c). For samples that reacted with both the 5-D-4 monoclonal antibody and type II collagen polyclonal antibodies, colocalization was observed in the juxtanuclear cytoplasm. Numerous other vesicles throughout the cytoplasm exhibited immunoreactivity with only the type II collagen polyclonal antibodies and not with the 5-D-4 monoclonal antibody (Fig. 4a, b). For samples that reacted with both 5-D-4 and the polyclonal antibodies to CSPG monomer, colocalization was observed in the perinuclear cytoplasm. The keratan sulfate antibody did not stain the uniformly sized vesicles that reacted intensely with CSPG monomer polyclonal antibodies (arrowheads, Fig. 4c, d).

Localization of Lectins

Intracellular compartments also were characterized using fluorescent-labeled lectins (see Introduction). As shown in Figure 5, wheat germ agglutinin, which binds to N-acetyl-D-glucosamine and which should stain the Golgi, reacted only with the perinuclear cytoplasm (arrows, Fig. 5a, e). In contrast, concanavalin A, which binds to D-mannose and which should stain the rough endoplasmic reticulum, reacted with vesicles distributed throughout the cytoplasm and not with the perinuclear region (Fig. 5b, c).

Lectin reactivity was compared with immunoreactivity. Vesicles distributed throughout the cytoplasm reacted both with concanavalin A and with type II collagen antibodies (Fig. 5c, d). Perinuclear staining was observed for type II collagen antibodies but not for concanavalin A (arrows, Fig. 5c, d). Colocalization of wheat germ agglutinin and antibodies against CSPG monomer was observed in the juxtanuclear cytoplasm (arrows, Fig. 5e, f). The vesicles that stained intensely with CSPG monomer antibodies did not react with wheat germ agglutinin (arrowheads, Fig. 5e, f).

DISCUSSION

The characterization of intracellular compartments involved in the synthesis and processing of complex cartilage matrix molecules such as CSPG and type II collagen has been facilitated by a combined analysis using polyclonal antibodies, selected monoclonal antibodies, and labeled lectins for double localization reactions. The following view of chondrocytes in culture, summarized in Table I, emerges from this analysis. Although chondrocytes react with antibodies to both type II collagen and CSPG, the intracellular distributions of the two antigens differ considerably. Intracellular type II collagen precursors are contained in vesicles throughout the cytoplasm and, to some extent, in the perinuclear cytoplasm. It is likely that the vesicles distributed throughout the cytoplasm correspond to elements of the rough endoplasmic reticulum, as concanavalin A is colocalized in these vesicles (see Introduction). In contrast to type II procollagen, precursors of CSPG monomers are relatively concentrated in the perinuclear cytoplasm (see below). It is likely that the perinuclear cytoplasm corresponds to the Golgi because wheat germ agglutinin and the keratan sulfate monoclonal antibody are each colocalized there. In some chondrocytes, CSPG precursors also are concentrated in several discrete vesicles per cell. Presumably, these other vesicles which contain CSPG precursors are pre-Golgi or early Golgi compartments, as they react neither with wheat germ agglutinin nor with the keratan sulfate monoclonal antibody. It may be significant to the synthesis, processing, or assembly of CSPG that these vesicles contain link protein [30] as well as CSPG monomer precursors and do not contain detectable type II collagen.

Our characterization of subcellular compartments was accomplished in part by the localization of specific lectins (see Introduction). Reactivity with labeled lectins has been used in many studies for the identification of subcellular compartments. As shown, concanavalin A and wheat germ agglutinin each reacted with different intracellular constituents. Vesicles distributed throughout the cytoplasm, but not in the perinuclear region, reacted with concanavalin A, and therefore may be considered rough endoplasmic reticulum. In contrast, the perinuclear cytoplasm reacted with wheat germ agglutinin and may be considered Golgi.
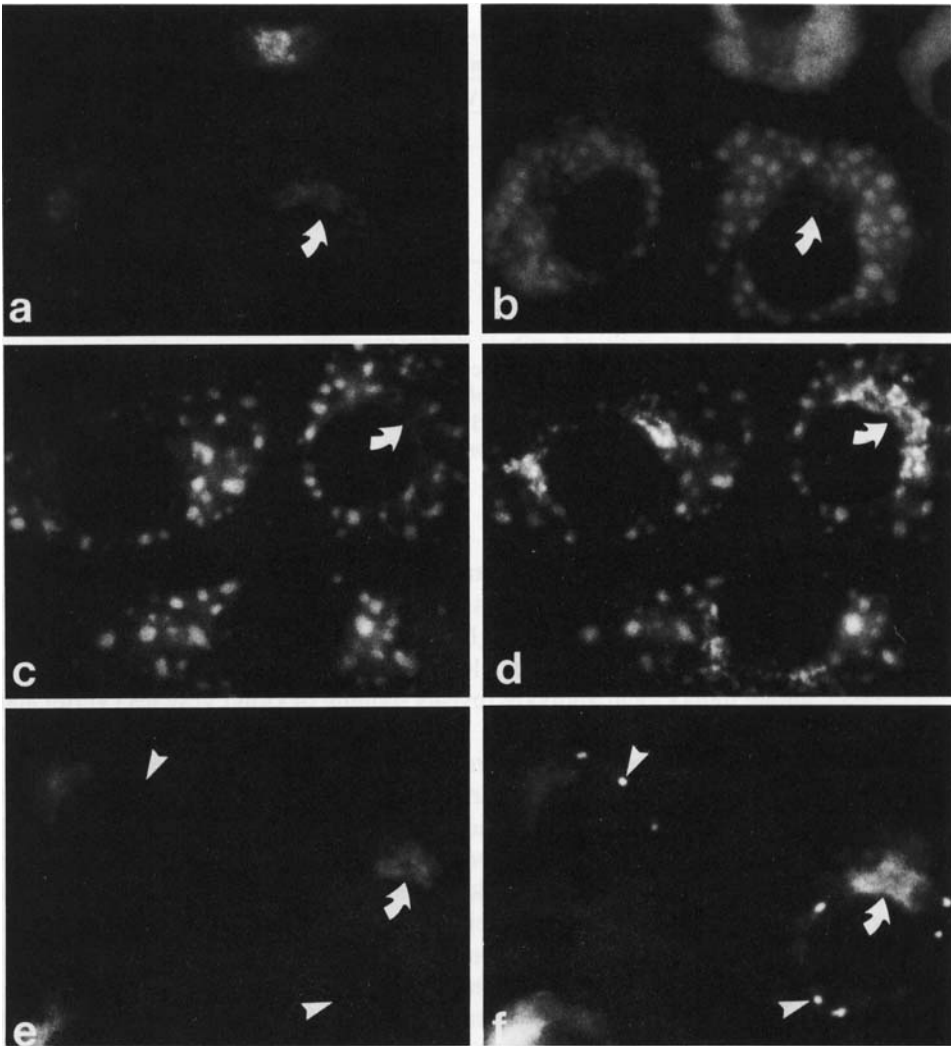
Fig. 5. Lectin localization and immunofluorescent staining of chondrocytes. Chondrocytes were grown in culture for 5 days, hyaluronidase-digested, fixed, and prepared for lectin (a,b) or lectin and antibody (c–f) localization as described in Materials and Methods. For each pair of micrographs, the field shown on the left is identical to the field shown on the right. The double-stained sample shown in a and b was incubated with TRITC-coupled wheat germ agglutinin and FITC-coupled concanavalin A. The double-stained sample shown in c and d was incubated with TRITC-coupled concanavalin A, guinea pig anti-type II collagen, and FITC-coupled goat IgG anti-guinea pig IgG. The double-stained sample shown in e and f was incubated with TRITC-coupled wheat germ agglutinin, guinea pig anti-CSPG monomer, and FITC-coupled goat IgG anti-guinea pig IgG. Note that the wheat germ agglutinin (a,e) stained only the perinuclear cytoplasm (arrows), while concanavalin A (b,c) stained vesicles throughout the cytoplasm but did not stain the perinuclear cytoplasm (arrows, b,c). Type II collagen antibodies (d) stained both the vesicles distributed throughout the cytoplasm which also stained with concanavalin A (c) and the perinuclear cytoplasm which did not react with concanavalin A (Arrows, c,d). Antibodies to CSPG monomer (f) stained the perinuclear cytoplasm which also reacted with wheat germ agglutinin (arrows, e,f) and stained several additional cytoplasmic vesicles which did not stain with wheat germ agglutinin (arrowheads, e,f). Magnifications (a,b,c,d), ×1600; magnifications (e,f), ×1800.

TABLE I. Localization of Lectins and Antibodies in the Characterization of Cytoplasmic Compartments of Chondrocytes*

Reactivity of cytoplasmic compartments	Lectin		Antibodies				Assigned organelle[b]
	Con A	WGA	II Col	CSPG	Ln[a]	KS	
1. Vesicles throughout cytoplasm outside perinuclear region	+ +	−	+ +	+	+	−	RER
2. Few 500–700 nm cytoplasmic vesicles (usually 0–4 per chondrocyte)	c	−	−	+ +	+ +	−	?
3. Perinuclear cytoplasm	−	+ +	+/−	+ +	+	+ +	Golgi

*Staining is described as follows: (−) none detectable; (+) weak but detectable; (+ +) intense.
[a]Data summarized from reference 30.
[b]Organelle assignments are based on lectin and antibody staining properties.
[c]The reactivity of "Compartment 2" vesicles with concanavalin A has not been determined conclusively. "Compartment 2" vesicles, characterized on the basis of staining with antibodies to the precursors of link protein and CSPG monomer and lack of staining with type II collagen antibodies, have been difficult to locate with certainty among the numerous vesicles that stain with concanavalin A.

Other observations support the contention that the perinuclear regions correspond to the Golgi. The present authors have demonstrated that the monoclonal antibody that recognizes keratan sulfate glycosaminoglycans reacts exclusively with the juxtanuclear cytoplasm. Preliminary electron microscopic immunolocalization of this keratan sulfate monoclonal antibody reveals staining of Golgi vesicles (Vertel and Weber, unpublished results). In another study, the present authors reported that different monoclonal antibodies, which recognize chondroitin sulfate glycosaminoglycan determinants, also stain only the perinuclear cytoplasm [29]. Cell fractionation studies [14,15,17] and autoradiographic analyses [12,13] have previously documented the substantial post-translational elongation of sulfated glycosaminoglycan chains in the Golgi. A perinuclear Golgi has been described in electron microscopic studies of cultured chondrocytes [39]. Recently, Pacifici et al [32] reported radioactive sulfate incorporation and the immunolocalization of CSPG antibodies in the perinuclear regions of vertebral chondrocytes grown in similar monolayer cultures.

Differences were observed in the intracellular distribution of type II procollagen and CSPG monomer precursors within the same cells. Localization of type II procollagen in vesicles throughout the cytoplasm (eg, rough endoplasmic reticulum) contrasted strikingly with the accumulation of CSPG monomer in the perinuclear cytoplasm (eg, Golgi). Pacifici et al [32] reported similar findings in their immunofluorescence studies of vertebral chondrocytes. Although in this report a predominance of immunolocalized type II procollagen throughout the cytoplasm was noted, some staining of the perinuclear cytoplasm was detected. In contrast, Pacifici et al [32] reported no perinuclear staining with their type II collagen antibodies. Thus, type II procollagen and CSPG precursors appear to follow the same route for secretion through the Golgi, even though they are accumulated in the rough endoplasmic reticulum or Golgi to a greater or lesser extent. Whether they are in fact contained in the same vesicles within that Golgi compartment is an issue that cannot be resolved using light microscopic analysis. However, electron microscopic studies of chondrocytes have reported the presence of fibrous material (presumably type II collagen-

containing) and granular material (presumably proteoglycan) often in the same secretory vacuoles and sometimes in different vacuoles [4,40,41].

The accumulation of intracellular collagen precursors has been documented previously for cells grown in culture without ascorbate supplements [8,42]. In another study [31], ascorbate was added to the culture medium of cells grown for several days without ascorbate and the clearing of accumulated type II procollagen was followed out of the rough endoplasmic reticulum and through the Golgi within a few hours. Apparently, the relative intracellular distribution of type II procollagen is dependent upon ascorbate levels and perhaps dependent upon other physiological conditions as well. As discussed above, the relative intracellular accumulation of type II procollagen and CSPG precursors may be different, but both of these cartilage matrix products appear to be secreted through the Golgi.

The perinuclear accumulation of precursors to CSPG monomer was observed consistently. A comparable pattern was obtained for the immunolocalization of CSPG precursors using the rat monoclonal antibody S103L [29]. Because this monoclonal antibody reacts with a single antigenic determinant present on 1) completed CSPG [43], 2) cell-free-translated CSPG core protein [38], and 3) CSPG precursors synthesized by cartilage cells in culture [19], it was assumed that the staining observed for S103L, and for the polyclonal antibodies used in the present study, accurately reflects the distribution and local concentrations of CSPG precursors. Kinetic studies of CSPG synthesis using radioactive precursors demonstrate that once glycosylation and sulfation has begun CSPG monomers are rapidly cleared from the Golgi [44,45]. Other analyses show that the CSPG precursor, without sulfated glycosaminoglycan chains, exhibits a relatively long transit time within the cell [18,46]. Based on these data, one might expect to find the accumulation of CSPG precursors in the rough endoplasmic reticulum (or perhaps in another non-Golgi compartment). Yet, immunolocalization results show unequivocally that CSPG precursors are accumulated in the perinuclear Golgi and that relatively less precursor is detected in the rough endoplasmic reticulum. This apparent discrepancy may reflect differences in the concentration of CSPG precursor molecules in different cytoplasmic compartments. In fact, more CSPG precursor molecules may be present in the rough endoplasmic reticulum; but, because the Golgi occupies a much smaller volume of the cytoplasm than does the rough endoplasmic reticulum, the relative staining intensity may be greater in the perinuclear Golgi. However, the possibility cannot be excluded that CSPG precursors are concentrated in the perinuclear cytoplasm because there exists a rate-limiting step in the processing of intracellular transport of CSPG within the Golgi prior to glycosaminoglycan chain elongation.

The vesicles that contain high concentrations of precursors of CSPG monomer and link protein, but which apparently do not contain type II collagen, are intriguing. These vesicles stained intensely with antibodies that react with all forms of CSPG monomer precursors (including the unmodified, cell-free-translated core protein) but did not stain with antibodies that recognize either keratan sulfate or the chondroitin sulfate glycosaminoglycan determinants [29]. Results of the present study strongly suggest that the vesicles correspond to a subcellular fraction that is a pre-Golgi or early Golgi compartment, as the proteoglycan precursors they contain have not been modified by Golgi-mediated, sulfated glycosaminoglycan additions. The presence of link protein and CSPG monomer precursors and the absence of type II collagen in

these vesicles suggest that they play a role in the intracellular transport or biosynthetic processing of proteoglycans and not the other major cartilage matrix molecule. Kimura et al proposed that link protein and CSPG monomer become associated with each other prior to secretion [46]. Perhaps the vesicles observed in this study are involved in the intracellular assembly of CSPG monomer and link. Alternatively, perhaps the vesicles are part of a degradative pathway for newly synthesized proteoglycan precursors. The variable frequency of occurrence of the vesicles among different cells in the same culture and among chondrocytes from different cultures may imply that they perform a regulatory function in proteoglycan synthesis and processing. More work is needed to establish the function of these vesicles in proteoglycan biosynthesis.

A variability in staining patterns for type II procollagen and CSPG precursors was noted among cells within the same culture. For example, some cells contained type II procollagen only in the perinuclear cytoplasm, and others contained type II collagen throughout the cytoplasm. Some chondrocytes contained several vesicles with concentrated CSPG precursors, whereas other cells contained very few or none. These differences may well reflect differences in the biosynthetic or differentiative states of individual cells. When cells are examined at the level of the individual in this way, differences can be observed that would not be detected by biochemical studies that examine populations of cells. The study of individual variability within populations of cells should provide a useful view to complement other analyses of biosynthesis.

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